Tartrate-Resistant Acid Phosphatase Deficiency in the Predisposition to Systemic Lupus Erythematosus

Jie An[*,1], Tracy A Briggs[*,#,2], Audrey Dumax-vorzet[2,3], Marta Alarcón Riquelme[4], Nalini Agrawal[1], Alex Belot[5], Michael Beresford[6], Ian Bruce[6], Laurence Chaperot[7] , Joel Plumas[7], Gillian I Rice[2], Tim Vyse[8], Alice Wiedeman[1], Yanick J Crow[,2,9], Keith B Elkon[#,1]

[1]Division of Rheumatology, Department of Medicine & Immunology, University of Washington, Seattle, WA98195, USA.
[2]Manchester Academic Health Science Centre, University of Manchester, Genetic Medicine, Manchester, UK.
[3]School of Medicine, Keele University, David Weatherall building, Keele University campus, ST5 5BG, UK.
[4]Marta address
[5]Alex address
[6] Ian address
[7]INSERM U823/UJF/EFS, Immunobiology & Immunotherapy of Cancers, La Tronche, France.
[8]Tim address
[9]Institut Imagine, Laboratory of Neurogenetics and Neuroinflammation, 24 boulevard du Montparnasse, Paris, 75015, France.

*These authors contributed equally to this work. [#]Corresponding authors

Abstract

Objectives: Biallelic mutations in *ACP5*, encoding tartrate-resistant acid phosphatase (TRAP) cause the immuno-osseous disorder Spondyloenchondrodysplasia, characterized by autoimmune disease, including systemic lupus erythematosus (SLE) and a type I interferon signature. Our aims were to determine the consequences of TRAP deficiency in human immune cells, identify the substrates of TRAP and assess whether *ACP5* mutations are enriched in 'idiopathic' SLE.

Methods: TRAP substrates were queried by yeast-2-hybrid screen in a human cDNA macrophage library and interaction with a candidate substrate, osteopontin (OPN), was confirmed by confocal microscopy and immuno-blot analysis. The effect of TRAP knockdown on OPN phosphorylation (using Biomol green assay and liquid chromatography-tandem mass spectrometry) and expression of selected cytokines (using ELISA or qPCR) was investigated. Sanger and targeted panel sequencing of *ACP5* was undertaken in patients with lupus and matched controls and phosphatase activity of genetic variants was studied.

Results: TRAP and OPN co-localized and interacted in human macrophages and plasmacytoid dendritic cells (pDCs). *In vitro* studies using recombinant human proteins showed that TRAP dephosphorylated at least three serine residues on OPN. Furthermore, OPN was hyperphosphorylated in TRAP knock down THP1 cells compared to controls. Knock down of TRAP expression in a pDC line showed increased nuclear translocation of IRF7 and P65, with resultant increased expression of interferon-stimulated genes, IL-6 and TNF following TLR9 stimulation. A significant excess of heterozygous *ACP5* missense variants was observed in lupus patients compared to controls ($p=0.04$) and *in vitro* experiments revealed a significant reduction in TRAP activity in over 60% of variants.

Conclusions: Our findings indicate that TRAP and OPN co-localize, and that OPN is a substrate for TRAP in immune cells. TRAP deficiency in pDCs leads to increased interferon-alpha production, providing at least a partial explanation for how *ACP5* mutations cause lupus in the context of Spondyloenchondrodysplasia. Detection of *ACP5* missense variants in a cohort of SLE patients suggests that impaired TRAP function may increase the susceptibility to idiopathic lupus.

Tartrate-resistant acid phosphatase (TRAP) is a member of the purple acid phosphatase family and is also referred to as type 5 acid phosphatase. It is predominantly expressed in cells of monohistocytic lineage, including osteoclasts, macrophages and dendritic cells (DCs){Minkin, 1982 #1;Hayman, 2001 #2}{Hayman, 2000 #18}. There are two isoforms of the TRAP enzyme: TRAP5a and TRAP5b, with TRAP5b being produced by post-translational modification of TRAP5a. TRAP5b is the major isoform of TRAP secreted by osteoclasts, and TRAP5b activity has been shown to correlate with osteoclast number and activity in the serum, in both rat and human studies {Atlanto, 2003 and/or Rissanen 2008} {Chu, 2003 #17}{Henriksen, 2007 #21}. In contrast, macrophages and DCs are believed to secrete TRAP5a as the predominant isoform, and TRAP5a is a nonspecific marker for macrophage activation {Janckila, 2009 #19}{Hayman, 2008 #20}. Most studies of TRAP function relate to its role in the osteoclast, where extracellular TRAP has been strongly implicated in the regulation of osteoclast attachment and migration, particularly via the dephosphorylation of osteoclast-secreted osteopontin (OPN) {Ek-Rylander, 1994 #3}. OPN is a highly phosphorylated, multifunctional glycoprotein that is secreted into biological fluids by many cell types including osteoclasts, macrophages and T cells {Inoue, 2011 #6}. OPN is known to be a key protein in bone mineralization, and it is thought that phosphorylated OPN facilitates attachment of the osteoclast to the resorbing bone matrix. Consequently, OPN dephosphorylated by secreted TRAP leads to osteoclast release and migration {Ek-Rylander, 1994 #3}.

Previously, we and others reported that biallelic mutations in gene *ACP5*, which encodes TRAP, results in a rare pediatric disorder named Spondyloenchondrodysplasia (SPENCD) {Briggs, 2011 #4;Lausch, 2011 #5}. Patients with SPENCD demonstrate a skeletal dysplasia reminiscent of that observed in the *ACP5* knockout mouse {Hayman, 1996 #22}. Interestingly, however, patients also manifest a variable neurological and autoimmune phenotype. These autoimmune features include ANA and anti-dsDNA autoantibodies, autoimmune thrombocytopenia purpura and systemic lupus erythematosus (SLE). Patients with SPENCD consistently demonstrate an overexpression of interferon (IFN)-stimulated genes (ISGs) in whole blood, an interferon signature, although the link from TRAP deficiency to IFN signaling remains unexplained.

Initial studies have implicated OPN as potentially relevant to the pathology of SPENCD {Briggs, 2011 #4;Lausch, 2011 #5}. OPN, also known as ‘Early T-cell Activation Factor’ {Patarca, 1993 #7}, is reported to be involved in diverse immune processes such as macrophage activation, inflammation and leukocyte recruitment, many of which are phosphorylation-dependent. It also plays a critical role in the efficient development of Th1 immune responses in T cells. Of note, polymorphisms in *OPN* and increased serum OPN levels have been associated with elevated interferon-alpha (IFN-α) levels in individuals with SLE {D'Alfonso, 2005 #8, Trivedi, 2011}. In the mouse OPN has been shown to be integral to IFN-α production in the plasmacytoid dendritic cell (pDC), a major source of type I IFN {Shinohara, 2006 #9}.

Studies to date have highlighted SPENCD as a rare Mendelian cause of lupus and suggest a previously unrecognized association between TRAP, OPN, and IFN-α metabolism. The aim of our research was to elucidate the detailed cellular pathways linking these molecules, and to understand how a loss of TRAP activity predisposes to autoimmune disease, particularly SLE.

Materials and Methods

Confocal microscopy

Human pDCs purified from PBMCs or a pDC line were stained on polylysine slides in a PAP circle of 100ul PBS containing 100K cells. Human macrophages derived from monocytes were stained in 4-chamber plates on Day 5 (which was plated at 0.5M cells/ml, i.e. per chamber on Day 0.) TRAP, OPN, IRF7 and NFkB were detected with anti-TRAP 5a rabbit sera {Lang, 2005}, mouse monoclonal anti-OPN antibody (Novus Biologicus)(for TRAP studies), Rabbit polyclonal anti-IRF7 and anti-P65 (Santa Cruz) respectively. Followed by secondary FITC labeled donkey anti-rabbit IgG (TRAP, IRF7, P65) and AF-555 donkey anti-mouse (OPN). Nuclei were stained with DAPI. Stained cells were viewed with a Zeiss LSM 510 confocal microscope with a 1.4 NA 63 x oil immersion lens and images were analyzed using the Image J.

RNA and cDNA preparation and quantitative real-time PCR

Total RNA was isolated from pDCs using the RNeasy mini kit (Qiagen, Valencia, CA). cDNA was synthesized using 100 ng RNA with the high-capacity cDNA RT-kit with random primers (Applied Biosystems, Foster City, CA). Reactions in duplicate were run on an ABI StepOne Plus using the primers: TRAP, IFN-α, IL6, TNF (primer sequences available on request). A two-stage cycle of 95°C for 15 s and 60°C for 1 min was repeated for 40 cycles followed by a dissociation stage. Threshold cycle values were set as a constant threshold at 0.2, and fold changes in gene expression were then calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot

Cells were lysed in lysis buffer composed of 0.5% NP-40 in 1X TBST, and 20 μg protein from each sample was loaded onto SDS-PAGE for western blot. The dilution of anti-TRAP was 1:1000 (USB, USA) and the dilution of anti-OPN was 1:1000 (R&D System, USA). Signals were detected with the ECL detection system and film (GE Healthcare, Piscataway, NJ, USA). Quantitation was carried out using BioRad imaging system (BioRad, USA) with normalization against the intensity of β-Actin. Quantitative infrared western blots were undertaken on *ACP5* variant homozygous HEK293 transiently transfected cells using primary mouse anti-HA (Sigma-Aldrich) and rabbit anti-tubulin (Sigma), with secondary IRDye 800CW goat anti-mouse (LICOR Biosciences) and IRDye 680RD goat anti-rabbit secondary IgG at 1/5000 (LICOR Biosciences). HA tag antibody was used, rather than TRAP antibody to exclude potential interference of point mutations with epitope recognition. Quantitation of bands was performed using Odyssey analysis software (LICOR Biosciences).

Yeast-two-hybrid screen.

A yeast-two-hybrid screen was performed by Hybrigencis services (www.hybrigenics-services.com) using an N-LexA-ACP5-C fusion protein and an N-Gal4-ACP5-C fusion protein on a Human Macrophage cDNA library from monocyte-derived macrophages from healthy donors. The N-Gal4-ACP5-C fusion yielded 36 positive clones, which were assessed for interactions and categorized as A to E according to predicted confidence in results.

Phosphorylation identification by mass spectrometry

TRAP knockdown THP1 cells were differentiated into macrophage-like cells by PMA (20nM) stimulation O/N. Cells were lysed with 6M Urea in 50mM Ammonium Bicarbonate. Proteins were reduced by TCEP for 1 hour at 37°C and alkylated with Iondoactamide for 1 hour at room temperature in the dark. Proteins were digested by Trypsin at 1:50 (Enzyme: Protein) ratio O/N at 37°C . The peptides were then washed three times and desalted by C18 columns. Phosphopeptides were enriched by TiO2 column and desalted by Graphite columns according to the manufacturer's instruction. Phosphopeptides were analyzed by UPLC (Waters, USA) coupled with OrbiTrap Fusion mass spectrometer (Thermal Scientific, USA). This experiment was performed four times.

Phosphate detection by BioMol Green

1ug TRAP and 1ug OPN were incubated together at 37°C O/N. The free phosphate released from OPN was measured by BioMol Green assay as per manufacturer's instructions (Enzo Life Sciences). Phosphate standards (Serial dilution from 2nmol to 0.031nmol) were used for the standard curve to quantify the released free phosphate.

Subjects

DNA samples where collected from patients with confirmed lupus as defined by ACR criteria {Tan}, in addition to matched controls. Control samples were obtained from 92 Swedish, 189 Portuguese, 61 British and 187 mixed European individuals. Lupus samples were obtained from 240 Swedish adults, 162 Portuguese adults, 129 pediatric and 352 adult British patients and 92 Argentinian adults. Consents and ethical approval was in place in each research group from which samples were obtained.

Sequence analysis

Genomic DNA was obtained from the clinicians of the subjects described above.

- Sanger sequencing

PCR amplification of all coding exons of *ACP5* was performed (sequences available on request). Purified PCR amplification products were sequenced using dye-terminator chemistry and electrophoresed on an ABI 3130 (Applied Biosystems) capillary sequencer. Mutation description is based on the reference complementary DNA (cDNA) sequence NM_ 001111035, with the ATG initiation site situated at the beginning of exon 4 and the termination codon in exon 7. The pathogenicity of variants was analyzed using Alumut, SIFT, PolyPhen and in the context of the crystal structure {Strater, 2005}. Minor allele frequency was assessed using the Exome Aggregation Consortium (ExAC) database

(exac.broadinstitute.org/).

- Next generation targeted panel sequencing

Targeted enrichment and sequencing were performed on 3 μg of DNA extracted from the peripheral blood of 85 individuals with pediatric SLE. Enrichment was undertaken using the SureSelect Human All Exon kits following the manufacturer's protocol (Agilent Technologies), and samples were paired-end sequenced on an Illumina HiSeq 2000 platform. Sequence data were mapped using Burrows-Wheeler Aligner using the hg18 (NCBI36) human genome as a reference. Data from 200 selected candidate lupus genes, including for *ACP5*, was extracted. Variants were called using SOAPsnp and SOAPindel (from the Short Oligonucleotide Analysis Package) with medium stringency. Variants were confirmed with Sanger sequencing as above.

Transient constructs.
Wild type human ACP5 cDNA with in frame Strep or HA tag was cloned into pcDNA3.2/GW/V5/D-TOPO vector (Invitrogen) and site-directed mutagenesis was performed to introduce individual point mutations into the ACP5-HA followed by confirmatory Sanger sequencing. The pcDNA3.2/GW/V5/D-TOPO without any ACP5 cDNA insert (empty vector) was used as a control. HEK293 cells were transfected O/N with 4 ug plasmid DNA using 10 ul Lipofectamine 2000 (ThermoFisher Scientific) according to the manufacturer's instructions. Cell supernatant and lysate was collected and protein concentration in lysates was determined using BCA assay (Pierce).

PNPP assay.
P-nitrophenyl phosphate assay was undertaken as previously described {Ljusberg et al. Biochem. J 343, 63-69, 199; Lang et al Cell.Mol.Life Sci 62: 905-918, 2005}. pNP concentration was normalized using protein concentration in the cell lysate. T tests were carried out using SPSS 20 (IBM SPSS Statistics 20).

Results

TRAP colocalizes and physically interacts with OPN in pDCs and macrophages.
Since OPN is a substrate for TRAP in osteoclasts {Andersson, 2003 #23}, we asked whether this was also the case in macrophages and pDCs . As shown in Fig. 1A, OPN and TRAP co-localized both in a pDC line Gen 2.2 {Chaperot, 2001 #12} and primary monocyte-derived macrophages as determined by confocal microscopy (Fig.1A). We also performed organelle studies and determined that both OPN and TRAP were localized in the golgi apparatus, as would be expected, as both proteins are secreted (data not shown).

To examine whether OPN and TRAP interact physically, we performed a yeast-two-hybrid screen in a human macrophage cDNA library. Whilst there were no category A-C (high confidence) interacting partners of TRAP identified, six category D (moderate confidence) interacting partners were demonstrated, including OPN, a golgi processing protein COG1, the transcription factor USF2, and three genes of unknown name or function. We confirmed OPN

as an interacting partner by over-expressing OPN and TRAP in HEK293 cells (Suppl. Fig.1) and performing immuno-blot analysis. TRAP was co-precipitated when OPN was immunoprecipitated (Fig.1B upper panel), whilst in the reciprocal experiment, OPN was co-precipitated with TRAP (Fig.1B lower panel). To verify that this interaction occurred in primary cells, we observed that when TRAP was immunoprecipitated in monocyte-derived macrophages, OPN was readily detected on the western blot (Fig.1C). Since the signal obtained with anti-OPN was much stronger than that precipitated by anti-TRAP, these results suggest that only some of the OPN is associated with TRAP. In summary, the yeast-two-hybrid and co-precipitation data, together with the confocal studies, indicate that OPN and TRAP interact with each other and that OPN is a substrate for TRAP in some human immune cells.

OPN is a substrate of TRAP *in vitro*.

OPN has the potential to be extensively modified by alteration of its phosphorylation state, as there are a number of serine/threonine phosphorylation sites distributed throughout the protein. The degree of phosphorylation varies depending upon the source of the OPN. For example, human and bovine milk OPN contain 32 and 28 serine/threonine phosphorylated sites respectively. The degree of phosphorylation of other forms of OPN, such as rOPN, is less certain {Wang, 2008; Christensen, 2012}. Since the degree of phosphorylation affects gel electrophoresis mobility {Ek-Rylander, 2010 #10}, we examined the effect of recombinant TRAP (rTRAP) on recombinant OPN (rOPN) migration by SDS-PAGE. As shown in Fig.2A, incubation with rTRAP altered the apparent molecular weight of rOPN from approximately 62 to 58KDa consistent with partial dephosphorylation of OPN. To assess more directly whether human OPN is a substrate for TRAP, rOPN was incubated with rTRAP. The amount of free phosphate liberated (determined using Biomol green assay) increased significantly when rOPN and rTRAP were incubated together compared to all other control conditions (Fig.2B). To determine the residues at which TRAP removed phosphates, we performed UPLC-LTQ-OrbiTrap mass spectrometry analysis of rOPN following incubation with rTRAP. Protein database search results revealed that TRAP consistently dephosphorylated two phospho-serine residues (Sp) in the peptide GKDSpYETSQLDDQSpAETHSHK and one phospho-serine residue (the first Sp) in the peptide ISHELDSpASpSEVN (Fig.2C).

Knockdown of TRAP results in an increase in IFN-α, IL6 and TNF production associated with increased nuclear translocation of IRF7 and NFkB.

Activation of TLR9 in pDCs leads to IRF7 and NFkB nuclear translocation resulting in the transcription of IFN-α**,** IL-6 and TNF {Gilliet, 2008 #11}. Since OPN was reported to associate with the TLR9-MyD88 signaling complex in pDCs in mice {Shinohara, 2006 #9} and we have shown that TRAP can associate with OPN and dephosphorylate it, we investigated the effects of knockdown (KD) of TRAP in the pDC line Gen 2.2 (hereafter referred to as pDC line) {Chaperot, 2001 #12}. We established a stable pDC cell line with a KD of TRAP of 66% (Fig.3A). Consistent with a role for TRAP in the regulation of OPN function, a significant increase in IFN-α concentration was observed in the TRAP KD pDC line compared to scrambled shRNA (control) following CpGA stimulation (Fig.3B).

Consistent with the increase in IFN-α, the expression of the ISGs IFI27 and CXCL10 were increased in the TRAP KD pDC line (Fig.3C). Of interest, stimulation of the TRAP KD pDC line with CpG also lead to increased production of the cytokines IL-6 and TNF (Fig.3D). No difference in cytokine production was observed in unstimulated cells.

To gain further insight into the mechanisms responsible for increased cytokine production, we examined the transcription factors IRF7 and NFkB downstream of TLR9 in pDC. Whereas there was no difference in the localization of IRF7 and NFkB in unstimulated cells (Suppl. Fig.2), we observed significantly more IRF7 (Fig.4A) and NFkB (Fig.4B) nuclear translocation in the TRAP KD pDC line, compared to the control pDC line, following CpG stimulation. These data demonstrate that TRAP plays a role in the regulation of IFN-α**,** IL6 and TNF cytokine production in human pDCs. Consistent with the *in vitro* data, a number of SPENCD patients showed significant elevation of IL-6 (but not TNF) expression in whole blood (Suppl. Fig.3), in addition to the elevated IFN-α data already reported {Briggs et al. 2011}.

OPN is hyperphosphorylated in TRAP KD compared to control THP1 cells

To further verify that TRAP regulates OPN function in pDCs, we sought to determine whether there is differential phosphorylation of OPN in TRAP KD versus control pDC line. Due to the low expression level of OPN in pDCs, attempts to detect phosphopeptides following CpG stimulation were unsuccessful. We therefore examined a monocytic control and TRAP KD THP1 cell line. Following PMA stimulation and differentiation to macrophage-like cells, TRAP KD THP1 cells consistently demonstrated an increased amount of hyperphosphorylated OPN quantified by LC-MS/MS, as compared to controls (Fig.5A). The hyperphosphorylated sites were within the same two peptides as the rOPN (Fig.2C), and two of three of the phosphorylated sites were the same (Fig.5B). Hyperphosphorylation of OPN in TRAP KD cells further indicated that OPN is a substrate for TRAP in immune cells.

***ACP5* heterozygous variants in SLE.**

In view of the high prevalence of lupus in SPENCD {Briggs, 2011 #4}, we sought to determine whether TRAP influenced susceptibility to idiopathic SLE. To address this question, we sequenced the four coding exons of *ACP5* in 890 SLE patients and 529 healthy matched controls by the Sanger method, and performed a target gene panel in a further 85 pediatric SLE patients. We observed an excess of rare heterozygous missense *ACP5* variants in the SLE patients (15/975) compared to controls (2/529) ($p=0.044$). The SLE patients with rare variants comprised 12 adults and 3 children of mixed ethnicity. We defined rare as a minor allele frequency <0.002 in the ExAC database, which includes data for *ACP5* on approximately 120,000 control population alleles. When *in silico* testing was performed, the missense residues were moderately to well conserved in mammalian species, and the majority were predicted to destabilize protein on *in silico* testing as shown in Supp. Table 1. Only one of the variants, Met264Lys, has previously been reported in a patient with SPENCD in the homozygous state.

To test whether the *ACP5* heterozygous variants identified in the SLE cohort could cause a

reduction in TRAP activity, we produced HEK293 cells expressing homozygous and subsequently heterozygous variant *ACP5* constructs. In the lysate and supernatant of cells transiently transfected with homozygous constructs, a significant reduction of TRAP activity (assessed using PnPP assay) was observed in seven out of eleven variants compared to wild type (Fig.6A). This correlated well with cytochemical staining of transiently transfected homozygous HEK293 cells (Supp. Fig.4). Quantitative western blot demonstrated that protein levels were minimally altered in the lysate (Fig.6B upper panel) and a Pearson's correlation coefficient of TRAP expression to activity (Supp. Fig.5) was low (R^2=0.1). This suggests that only around 10% of the variation in activity in the lysate could be attributed to variation in expression level. We therefore hypothesize that the origin of the majority of the variation in activity is not an absence of protein, but is due to an affect on catalytic activity. In contrast, in the supernatant, quantitative western blot demonstrated a reduction in protein levels in those variants in which activity levels were significant reduced (Fig.6B lower panel). The high Pearson's correlation coefficient between relative TRAP expression and activity (R^2=0.98) (Supp. Fig.5), suggests that over 97% of variation in activity can be attributed to variation in protein expression level. As quality control in secretory pathways is highly efficient at sifting through misfolded protein to ensure that only correctly folded active proteins are secreted, we propose that some variants may be misfolded and thus poorly secreted into the supernatant.

We next expressed five of the variants in the heterozygous state to more accurately simulate the situation in SLE patients. We chose to express four variants that demonstrated significantly reduced activity in the homozygous state, in addition to the Thr5Met variant for which *in silico* prediction was not possible since it lies outside of the reported crystal structure. Four of the heterozygous variants showed a reduction in TRAP activity, which was statistically significant in the lysate of two and the supernatant of three *ACP5* variant constructs. Western blot analysis was not possible in these cells due to the co-expression of WT and variant TRAP, however equal expression of both constructs was confirmed by qPCR. Of note, since shRNA knockdown of TRAP to ~30% expression was sufficient to cause ISG upregulation following pDC stimulation (see above), we hypothesize that a number of these rare heterozygous *ACP5* missense variants are functionally and clinically relevant due to reduced TRAP activity. Further assessments of sub-cellular localization and post-translational processing may identify further functional consequences of point mutations, especially those that do not appear to effect protein activity.

Discussion

In this study, we investigated the role of TRAP and OPN in innate immunity in humans with special relevance to SPENCD and the systemic autoimmune disease SLE. We found that TRAP co-localized and physically interacted with OPN in pDCs and in macrophages, and that OPN is a substrate for TRAP. When TRAP expression was reduced in pDCs, we observed that TLR9 stimulation caused an increased nuclear translocation of IRF7 and P65 with associated elevation in ISGs, IL-6 and TNF expression thus offering an explanation for the interferon signature in SPENCD patients. Our findings may be of relevance not only to the pathogenesis of SPENCD, but also to lupus susceptibility as, in a survey of SLE patients,

we demonstrated an excess of heterozygous *ACP5* missense variants, several of which display impaired catalytic activity.

To understand the relationship between TRAP deficiency and type I IFN production in SPENCD patients, the hypothesis that we explored in this study stems from the work of Shinohara *et al*. {2006} who reported that the association of OPN with the TLR9-MyD88 signaling complex was essential for IFN-α production in murine pDCs {Shinohara, 2006 #9}. However, in those studies, phosphorylation of OPN was not explored. Activation of the TLR9-MyD88 signaling pathway within pDCs has been shown to lead to both IRF7 and NFkB nuclear translocation resulting in the transcription of IFN-α, IL-6 and TNF {Gilliet, 2008 #11}. We established further evidence for a role of TRAP in the regulation of this pathway; specifically, when we knocked down TRAP expression in a pDC line, we observed that TLR9 stimulation caused increased nuclear translocation of both IRF7 and NFkB along with an elevation in IFN-α, IL6 and TNF, compared to control cell lines. These data are consistent with the clinical observation of significant elevation of IFN-α in SPENCD cases {Briggs, 2011 #4} and the elevated IL-6 levels detected in several patients.

We propose that the increased IFN-α production following CpGA stimulation in TRAP deficient pDCs is secondary to the action of the persistent (unregulated) action of the TLR9-MyD88-OPN signalosome, as illustrated in Supp. Fig.6. Thus, in TRAP deficiency, an absence of OPN dephoshorylation and deactivation results in persistent formation of the OPN-TLR9-MyD88 complex, with increased IFN-α production and a predisposition to autoimmune disease. Experimentally, we were not able to confirm this possibility as we could not assess OPN phosphorylation (due to limited substrate availability from even 50 million cells in our pDC KD line using LC-MS/MS). It therefore remains to be formally determined whether OPN is a physical component of the TLR9-MyD88 complex in human cells, or whether TRAP acts on a different or even multiple substrates in this pathway.

The function of TRAP has previously been explored in another myeloid derived cell, the osteoclast. Ultrastructural immunohistochemistry revealed that, similar to OPN, TRAP is localized to the resorption lacuna, where it may directly contact bone OPN in an acidic environment {Flores, 1992 #14}. Here, TRAP dephosphorylation of OPN facilitated osteoclast migration during bone resorption (XX). In TRAP deficient mice, delayed clearance of the microbial pathogen *Staphylococcus Aureus*, and a reduced population of macrophages in the peritoneal exudates was observed suggesting that TRAP may directly or indirectly influence recruitment of macrophage to sites of microbial invasion {Bune, 2001 #15}. Concurrently, *in vitro* studies showed that phosphorylation-dependent interaction of OPN with its receptor regulated macrophage migration and activation {Weber, 2002 #16}. Whether or not TRAP regulation of OPN influences macrophage recruitment in SPENCD is unknown.

Loss of TRAP activity causes SPENCD, and half of SPENCD patients fulfilled ACR diagnostic criteria for SLE whilst nearly all had positive anti-dsDNA and/or ANA antibody titers {Briggs, 2011 #4;Lausch, 2011 #5}. These findings suggest that TRAP might influence susceptibility to idiopathic SLE. Sequencing of *ACP5* gene in nearly 1000 SLE patients

demonstrated a significant excess of heterozygous *ACP5* missense variants in SLE patients compared to controls. In addition there was a reduction of TRAP activity related to over 60% of these variants in an *in vitro* transfection assay, indicating that impaired function of TRAP may play a role in the susceptibility to idiopathic lupus in a small proportion of patients.

In conclusion, our findings indicate that TRAP and OPN co-localize, and that OPN is a substrate for TRAP in immune cells. Significantly, TRAP deficiency in pDCs leads to increased IFN-α production, providing at least a partial explanation for how biallelic *ACP5* mutations cause SLE in the context of SPENCD. Detection of heterozygous *ACP5* missense variants in lupus patients suggests that impaired function of TRAP may play a role in the susceptibility to idiopathic lupus.

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References

Figure legends

Fig. 1. Colocalization and interaction of TRAP and OPN in pDCs and primary macrophage cells.

A, Confocal microscopy of TRAP (green) and OPN (red) with the overlay demonstrating partial colocalization in a pDC line Gen 2.2 (upper) and macrophages (lower). **B,** HEK293 cells were co-transfected with OPN and TRAP. After 48 hrs, cell lysates were made and target proteins immunoprecipitated (IP) with the antibodies as shown. Samples were resolved by SDS-PAGE and then immunoblotting (IB) performed with antibodies targeted to the antigens shown. Upper panel: anti-OPN IP followed by anti-TRAP IB; lower panel: anti-TRAP IP followed by anti-OPN IB. **C,** IP and immunoblotting were performed as in C except that the lysates were made from primary macrophages stimulated with CpG-B for 18hours. Key: The positive control was loaded in the first lane of each gel and IgG is the appropriate isotype control for each IP.

Fig. 2. OPN is a substrate for TRAP.

A, Human recombinant (rOPN, 200ng) was incubated with human recombinant TRAP (rTRAP,

200 ng) overnight and immunoblot (IB) performed with anti-OPN antibody as the detection antibody. **B,** Bovine milk OPN (bmOPN, 1µg) or rOPN (1µg) were incubated with rTRAP (1µg) overnight {EkRylander, 2010}. Free phosphates were quantified by BioMol green assay with phosphate as standards; bmOPN, rOPN, rTRAP or reaction buffer only were used for negative control for background signal. *p=<0.05. **C,** Human rOPN (500 ng) was incubated with rTRAP (500 ng) overnight. The reaction mixtures were trypsin digested and subjected to LC-MS/MS for the detection of phosphorylated peptides from OPN protein. The three phosphorylation sites (Sp: phoshorylated Serine) on two peptides of OPN which were found to be dephosphorylated by TRAP are depicted in red. The reaction was performed in duplicate.

Fig. 3. Knockdown of TRAP increases production of IFN-α, ISGs, TNF and IL6 in pDCs. Empty Vector, Scrambled shRNA or TRAP shRNA transfected Gen 2.2 pDCs were either unstimulated (panel A), stimulated with CpG-A (panels B-C) or CpG-B (panel D) for 16 hours. **A,** ACP5 mRNA expression was measured by q-PCR and normalized to housekeeping gene 18s mRNA expression, **p=<0.01. **B,** IFN-α cytokine production was quantified by ELISA. **C,** IFN-stimulated genes IFI27 and CXCL10 mRNA expression was measured by q-PCR and normalized to housekeeping gene 18s mRNA expression. **D,** TNF and IL6 cytokine production was quantified by ELISA.

Fig. 4: TRAP deficiency in pDCs increases nuclear translocation of IRF7 and NFkB. **A,** The pDC line, Gen 2.2, was stimulated with CpG-A for 1 hour and analyzed by confocal microscopy using DAPI (green) to stain the nucleus and an antibody to the transcription factor, IRF7 (red). A representative image is shown on the left and % of cells with IRF7 nuclear translocation post stimulation was quantified in TRAP KD pDCs compared to scrambled shRNA control, as shown on the right. **B,** Confocal microscopy of DAPI and NFkB (p65 subunit) in a representative image from the pDC line Gen 2.2 stimulated with CpG-B for 1 hour is shown on the left, with quantification of NFkB nuclear translocation in TRAP KD pDCs compared to scrambled shRNA control presented on the right. The % of cells with nuclear translocation post stimulation are shown in both cases in the graph.

Fig. 5. OPN is hyperphosphorylated in TRAP KD THP1 cells. THP1 cells were transfected with either scrambled shRNA or TRAP shRNA as in Methods and were differentiated to macrophage-like cells for 24 hours with PMA. . Cell lysates were subjected to trypsin digestion and the phosphopeptides were enriched by TiO2 column followed by mass spectrometry (LC-MS/MS). Acquired mass spectra were searched against the human protein database with phosphorylation as an alternative modification. Acquired OPN phosphopeptide counts were normalized to total peptides counts in the same mass spec run for the OPN phosphopeptides quantification between scrambled shRNA and TRAP shRNA samples. **A,** The abundance of phosphorylated OPN peptides in the TRAP shRNA compared to scrambled shRNA. **B,** The three specific serine residues in two peptides of OPN which were hyper-phosphorylated in the TRAP shRNA are depicted in red. The experiments were repeated four times.

Fig. 6: TRAP activity in supernatant and cell lysates obtained from HEK293 cells

transiently transfected with homozygous or heterozygous *ACP5* variants identified in a lupus cohort. TRAP activity measured by PnPP assay, was normalised to total protein concentration in the cell lysate. Data presented are mean values of n = 4-6 independent experiments, error bars: +/- SEM. **A,** TRAP activity in the cell lysate and supernatant in eleven homozygote variants compared to WT protein and empty vector (EV). **B,** Ten microgram of total cell lysate protein and an equal volume of supernatant were analysed by quantitative infrared western blot. Tubulin was used as both loading control in cell lysates and quality control; its absence in supernatants indicates that proteins in supernatant were secreted by intact cells. This is a representative example of n = 4 western blots. **C,** TRAP activity in the cell lysate and supernatant in five heterozygote variants compared to WT protein and empty vector (EV). Equal amounts of Strep-tagged WT and HA-tagged mutant ACP5 were transfected and expression of both constructs was confirmed by qPCR (data not shown).

Supplementary Figure legends

Supplementary Fig. 1. Overexpression of TRAP and OPN in HEK293 cells.
Immunoblot (IB) of TRAP (upper panel) or OPN (lower panel) proteins following overexpression in HEK293 cells transfected with OPN, or TRAP, or OPN and TRAP plasmids together as indicated in the legend above the figure.

Supplementary Fig. 2. Localization of IRF7 and NFkB in unstimulated TRAP deficient and scrambled pDCs.
The unstimulated pDC line, Gen 2.2, was analyzed by confocal microscopy using DAPI (green) to stain the nucleus and an antibody to IRF7 (upper panel) or NFkB (lower panel)(red). A representative image is shown for both antibodies in TRAP KD pDCs compared to scrambled shRNA control.

Supplementary Fig. 3. Interleukin 6 and TNF expression in SPENCD patients and controls. Quantitative reverse transcription PCR (qPCR) of IL-6 and TNFα expression in whole blood from 12 SPENCD patients (18 samples) compared to 24 healthy controls. Horizontal red bars show the median RQ value for each probe in each group. All values are shown for patients with biological replicates.

Blood was collected into PAXgene tubes and total RNA was extracted using a PAXgene RNA isolation kit (PreAnalytix). qPCR analysis was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems) and cDNA derived from 40ng total RNA. The relative abundance of target transcripts, measured using TaqMan probes for *IL6* (Hs00985639_m1) and *TNFA* (Hs99999043_m1), was normalized to the expression level of *HPRT1* (Hs03929096_g1) and *18s* (Hs999999001_s1) and assessed with the Applied Biosystems StepOne Software v2.1 and DataAssist Software v.3.01. For each probe, individual data were expressed relative to a single control calibrator. RQ is equal to $2^{-\Delta\Delta Ct}$ i.e. the normalized fold change relative to a control. Each value is derived from four technical replicates. Data was analyzed by Kruskal-Wallis test using Dunn's multiple comparison test.

Supplementary Fig. 4: TRAP activity measured by immunocytochemistry staining in the

homozygous *ACP5* variants identified in a lupus cohort.
TRAP hydrolyses naphthol AS-Bl phosphoric acid to naphthol AS-BI, this couples with fast garnet GBC forming insoluble maroon dye deposits at the site of activity. The intensity of the purple stain in the cells provides an indication about the level of TRAP activity. This is a representative example of n=3 independent experiments. Scale bar = 100 mm. Staining in the empty vector (A) and WT (B) were compared to that of the variants: Thr5Met (C), Arg46Trp (D), Phe141Val (E), Thr183Lys (F), Val208Met (G), Glu213Gln (H), Met264Lys (I), Arg269Trp (J), Arg272His (K) and His282Arg (L).

Supplementary Fig. 5: Pearson product moment correlation between activity and expression levels (relative to WT) in cell lysates and supernatants obtained from HEK293 cells transiently transfected with homozygous *ACP5* variants identified in an SLE cohort.
Mean catalytic activity (n = 4-6, measured by PnPP assay) relative to WT TRAP is represented on the horizontal axis while mean relative expression level (n = 4, assessed by infrared western blot) is represented on the vertical axis for all *ACP5* mutants analysed. The error bars represent the SEM. The line of best fit using Pearson's correlation is also displayed on the graph. **A,** The correlation between relative protein expression and activity in cell lysates was low (r^2=0.107, r = -0.328, n=12). **B,** In the supernatant, a very high level of correlation (r^2=0.977, r =0.989, n=12) was found between relative expression and activity.

Supplementary Fig. 6: Hypothesis for TRAP/OPN axis in the regulation of IFN-α in human pDCs.
Step 1 illustrates phosphorylation, and thus activation, of iOPN (intracellular OPN), perhaps by the inducing DNA ligand. Activated iOPN then forms a complex with TLR9 and MyD88 and, via IRF7, induces IFN-α production. Regulation of the pathway, as is shown in the upper panel of Step 2, is achieved by dephosphorylation of OPN by TRAP, with subsequent inhibition of IFN-α induction. The lower panel in Step 2, demonstrates the continued formation of the iOPN/MyD88/TLR signalosome with prolonged IFN-α production due to a deficiency of TRAP, as in SPENCD and possibly, some cases of SLE.

Supplementary Table 1. Rare missense heterozygote *ACP5* variants identified in the SLE cohort.